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Title: IL-1RA mediates TLR 3-induced inhibition of trophoblast adhesion to endometrial cells *in vitro*.

Running title: IL-1RA decreases embryo implantation.

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Abstract

ABSTRACT

Study question: Is IL-1RA involved in the TLR 3-induced inhibition of trophoblast cells' adhesion to endometrial cells *in vitro*?

Summary answer: IL-1RA mediates the TLR 3-induced inhibition of trophoblast cells' adhesion to endometrial cells *in vitro*.

What is known already: It is well documented that endometrial TLR 3 activation leads to impairment of trophoblast binding to endometrial cells *in vitro*. IL-1 receptor antagonist (IL-1RA) is known as an anti-implantation factor, as its injection significantly reduced implantation rates in mice by the effect on endometrial receptivity.

Study design, size, duration: Poly I:C was used as TLR3 specific ligand and endometrial cells were either treated or not with Poly I:C (treated versus control) *in vitro*. IL-1RA was applied to block IL-1 signal transduction. IL-1RA was knocked down by Accell Human IL1RN siRNA. Flagellin was used to stimulate TLR 5. SP600125 (JNK) was applied to inhibit the MAPK pathway. BAY11 -7082 was used to inhibit the NF-κB pathway. The experiments were performed in three replicates on three separate days.

Participants/materials, setting, methods: An *in vitro* assay was developed using RL95-2 (an endometrial cell line) and JAr (a trophoblast cell line) cells. Initially, the production of IL-1RA in RL95-2 cells in response to TLR 3 activation was measured. To determine whether the TLR 3-induced inhibition of trophoblast binding was mediated through IL-1RA: (i) we evaluated the effect of IL-1RA on the attachment of trophoblast cells to endometrial cells; (ii) we knocked down TLR3 induced IL-1RA gene expression by IL-1RA siRNA and evaluated trophoblast attachment to endometrial cells. Finally, to clarify through which pathway TLR 3-induced inhibition of trophoblast binding occurs: (i) activation of NF-κB and MAPK was detected by transfecting the cells with secreted placental alkaline phosphatase (SEAP) reporter plasmids bearing promoter sequences for each transcription factor; (ii) the inhibitors for NF-κB and MAPK were used to block signaling; (iii) it was then investigated whether addition of these inhibitors could restore the TLR 3-induced impairment of trophoblast attachment to the endometrial cells.

Main results and the role of chance: Our results showed that addition of Poly I:C to RL95-2 cells significantly increased the production of IL-1RA ($P < 0.05$). Addition of human recombinant IL-1RA to RL95-2 cells remarkably decreased the adhesion rate of trophoblast cells to endometrial cells ($P < 0.05$). In addition, suppression of TLR3-induced IL-1RA gene expression in RL95-2 cells significantly restored trophoblast cells attachment to endometrial cells in the presence of Poly I:C ($P < 0.05$).
Activation of MAPK was only induced by TLR 3 activation ($P < 0.05$). Of NF-kB and MAPK inhibitors, only MAPK's inhibitor could achieve restoration of spheroid adhesion to endometrial cells ($P < 0.05$).

Limitations, reasons for caution: This study has been only done *in vitro*. Future *in vivo* studies will confirm our data.

Wider implications of the findings: The findings of this study have a potential clinical application in introducing IL-1RA as one of the diagnostic infertility markers in the endometrium, which can affect the process of embryo adhesion at the time of implantation. Moreover, based on the novel data obtained in the current study, blocking and regulating the MAPK pathway by its inhibitors can be used as a new strategy to prevent and treat virus-induced infertility cases in ART techniques.

Study funding/competing interest(s): The authors have no conflict of interest to declare.

Key words: Toll-like receptor 3, infertility, IL-1RA, MAPK, embryo implantation.

Introduction

Despite many improvements in the assisted reproductive technologies (ART), implantation failure remains to be the major problem affecting the outcome of ART (Carver et al., 2003). Successful implantation relies on a high quality embryo, a receptive uterus and a series of tightly regulated interactions between the blastocyst and the endometrium. It has been shown that activation of the innate immune system in the female reproductive tract (FRT) in response to genital tract infections can affect this communication (Pellati et al., 2008, Dekel et al., 2010).

The innate immune system in the FRT recognizes infectious microorganisms through pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997, Medzhitov and Janeway, 2002). To date, 10 members of the TLR family have been identified in humans (TLR 1 to 10), each of which recognizes and binds to a specific ligand (Beutler, 2004). Among the various TLR members, TLR 3 recognizes double-stranded RNA and plays an important role in the recognition of infectious viruses (Jorgenson et al., 2005, Schaefer et al., 2005, Yu and Levine, 2011). Upon detection of viruses by TLR3, an intracellular

cascade of molecular reactions is triggered, which leads to stimulation of the transcription factors nuclear factor (NF-κB) and mitogen-activated protein kinases (MAPK). Stimulation of MAPK would in turn activate the activating protein (AP)-1 composed of the proteins Jun and Fos. NF-kB and AP-1 are trans-located to the nucleus after activation, which results in induction of type 1 interferon (IFN), pro-inflammatory cytokines and chemokines (Matsumoto et al., 2011).

There is a considerable body of evidence that TLR 3 is expressed in the primary uterine epithelial cells (Schaefer et al., 2005, Aflatoonian et al., 2007) and endometrial cell lines including ECC-1 (Schaefer et al., 2004), Ishikawa (Aboussahoud et al., 2010a) and RL95-2 (Jorgenson et al., 2005). It has also been shown that TLR 3 recognizes viruses such as cytomegalovirus (CMV) and herpes simplex virus 1 (HSV-1) (Matsumoto et al., 2011), whose strong association with female infertility has been shown in many studies (el Borai et al., 1997, Medvedev et al., 2009, Yang et al., 1995). A significant association between infertility and HSV positive test was observed in women after failed in vitro fertilization (el Borai et al., 1997). Similarly, it was found that seroprevalence and genital viral shedding of CMV were relatively high in infertile women (Yang *et* al 1995). Moreover, Inflammatory changes in reproductive organs in women with tubal-peritoneal infertility are determined by chronic herpesvirus infection (Medvedev et al., 2009). A significant association between CMV and HSV co-infection (Rasti et al., 2015) and HSV-2 infection alone (Kalu et al., 2015, Kapranos and Kotronias, 2009) and occurrence of spontaneous abortion have been found. Fetal CMV infection is also linked to congenital abnormalities such as central nervous system anomalies, hydrops fetalis and oligohydramnios, as well as orofacial clefts (Weichert et al., 2010).

Similarly, fetal HSV infection is associated with extensive brain damage, hemorrhage, and cystic encephalomalacia (Vasileiadis et al., 2003).

The effect of activation of some members of TLR family on embryo implantation has been shown before, with stimulation of TLR 2/6, 3 and 5 leading to impairment of trophoblast cells' attachment to endometrial cells *in vivo* and *in vitro* (Aboussahoud et al., 2010b, Sanchez-Lopez et al., 2014, Montazeri et al., 2015). Implantation failure is likely to be the major cause of infertility, thus deep insight into the molecular mechanisms that impact the process of embryo implantation in response to TLR 3 activation may provide new opportunities for improving the implantation rate in virus-induced infertility cases.

Emerging evidence suggests that in addition to adequate hormonal priming, successful embryonic implantation relies on an appropriate interaction between cytokines produced and received by the blastocyst and endometrium (Cross et al., 1994). The interleukin-1 (IL-1) system seems to be relevant to the implantation process (Kauma et al., 1990). The IL-1 family consists of two agonists, IL-lα and IL-1β (Dinarello, 1988), and an inhibitor, IL-1 receptor antagonist (IL-1RA). Two IL-1 receptors have been recognized: IL-1 receptor type I (IL-1R tI) (Sims et al., 1988) and IL-1 receptor typeII (IL-1R tII) (Horuk and McCubrey, 1989). The available information indicates that IL-1R tII is not functional and IL-1 signalling occurs exclusively via type I receptors (Sims et al., 1993).

The presence of the IL-1 system in human endometrium (Tabibzadeh and Sun, 1992, Kauma et al., 1990, Simon et al., 1995, Simon et al., 1993a, Simon et al., 1993b), human embryos (De los Santos et al., 1996) and embryo-maternal interface (Simon et al., 1994) has been

143 previously demonstrated. Furthermore, it has been shown that IL-1 expression significantly
144 increases at the late secretory phase, when the implantation of the embryo takes place
145 (Simon et al., 1993a). These evidences suggest that IL-1 system may have a pivotal role in
146 controlling and regulating the process of embryo implantation. IL-1RA can inhibit the binding
147 of IL1α and IL1β to IL-1R tI and its binding to the receptor does not result in signal transduction
148 (Bankers-Fulbright et al., 1996). IL-1RA is also regarded as an anti-implantation factor and as
149 Simon and colleagues have shown, IL-1RA prevented embryonic implantation in mice by α4,
150 αv and β3 integrin subunits expression alteration on the endometrial epithelium (Simon et
151 al., 1998).
152
153 In the current investigation we hypothesized that IL-1RA mediated TLR 3-induced impairment
154 of trophoblast adhesion to endometrial cells. We tested the validity of this hypothesis by
155 measuring the production of IL-1RA in RL95-2 cells in response to TLR 3 activation and
156 determined if IL-1RA has a negative effect on trophoblast adhesion to endometrial cells.
157 Furthermore, we investigated if suppression of IL-1RA production by IL-1RA siRNA in RL95-2
158 cells could restore the adhesion of JAr spheroids to the endometrial cells in the presence of
159 Poly I:C. The results obtained confirmed IL-1RA involvement in TLR 3-induced reduction of
160 trophoblast cells binding to endometrial cells. Finally we investigated the role of NF-κB and
161 MAPK pathways activation in mediating TLR3-induced impairment of trophoblast cells
162 adhesion to the endometrial cells.

Methodology

Cell lines and cell culture

RL95-2 was obtained from ATCC and was used to mimic endometrial cells. RL95-2 cells were cultured in T75 flasks at 37º C in DMEM (F12) HAM (Invitrogen, Paisley, UK), supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma, Poole, UK), 10% FCS (Invitrogen), 160 ng/ml Insulin (human recombinant insulin from Gibco (Invitrogen), catalog # 12585-014, and 2mM L-glutamine (Invitrogen), in 5% CO2 atmosphere until confluence was reached. The human choriocarcinoma cell line, JAr, was obtained from ATCC (catalog NO. HTB-144) and used as a model for trophoblast cells. JAr cells were grown in RPMI 1640 (Sigma), supplemented with 10% FCS (Invitrogen), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma), and 2 mM L-glutamine (Invitrogen). At confluence, the cells were washed with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS; Sigma) and harvested using trypsin-EDTA (Invitrogen). The cells were then incubated for 3 min, pelleted by centrifugation at 300 g for 4 min and the supernatant was discarded. The cells were diluted with 3 ml of media and suspended with pipetting 5-6 times in order to ensure a homogenised solution.

Ligands and inhibitors

Poly Inosinic Poly Cytidilic Acid (Poly I:C) (Invivogen, tlrl-pic, Toulouse, France), TLR 3 synthetic ligand was used to stimulate TLR 3 (Alexopoulou et al., 2001). IL-1 receptor antagonist (IL-1RA) (PeproTech, 200-01RA, London, UK) was applied to block IL-1 signal transduction. Flagellin was used to stimulate TLR 5 (Hayashi et al., 2001). SP600125 (JNK) was applied to inhibit the MAPK pathway (Bennett et al., 2001). BAY11 -7082 was used to inhibit the NF-κB pathway (Saraiva et al., 2005). All ligands and inhibitors used in the current study were obtained from Invivogen Company (Invivogen, Toulouse, France).

In vitro human implantation assay

The RL95-2 cells were cultured in T75 flasks until 100% confluence, cells were then harvested using trypsin-EDTA. The cells were counted and $3x10^5$ endometrial cells were cultured in each well of a 12-well plate. They were incubated at 37°C and 5% of CO2 for 4 days until confluence. The media were replaced every second day.

To create spheroids from JAr cells monolayers, 10^6 cells/ml were counted with a Haemocytometer, and cultured in 5 ml of RPMI 1640 media in 60 × 15 mm Petridishes (CellStar tissue culture dishes, Greiner Bio-One, GmbH/Germany) in a humid atmosphere containing 5% CO_2 at 37°C on a gyratory shaker (IKA MTS 2/4, Staufen, Germany), set at 300 rpm for 24 h.

Once the JAr spheroids were formed on the shaker, they were gently transferred onto each well of confluent RL95-2s in 12-well plates, and the co-culture was maintained in DMEM-F12 HAM, with supplements as mentioned above and incubated for 1 h at 37°C. The images of JAr spheroids and RL95-2s co-culture were captured by a Nikon DS-Fi1 camera (Nikon, Kingston Upon Thames, UK) connected to an inverted CKX41 fluorescent microscope (OLYMPUS, Tokyo, Japan).

Non-adherent spheroids were removed from the monolayer using an automatic horizontal shaker (Labman Automation LTD) to detach loosely bound or unbound spheroids. In brief, once the trophoblast spheroids were co-cultured with endometrial cells, the number of spheroids was counted under the microscope and each plate was placed on a shaker, which was set at 200 rpm for 4 min. The cells were washed with DPBS twice and then the number of attached spheroids was counted under the microscope. The results were expressed as the percentage of spheroids attached from the total number of spheroids used to initiate the co-incubation experiments. All the experiments were performed in three replicates.

RNA isolation and cDNA synthesis

For endometrial cell lines genomic studies, RL95-2 cells were washed with DPBS without Ca^{2+} and Mg^{2+} and one milliliter of TRIreagent (Sigma) was added onto the flask. Thereafter total RNA from cells was extracted following a standard protocol supplied by the manufacturer. Total RNA obtained from RL95-2 cells was treated three times with DNase I (DNA-freeTM, Ambion Austin, TX, USA) to remove genomic DNA contamination from the samples. First

strand cDNA synthesis was performed using oligo dT primers (Metabion, Martinsried, Germany) and reverse transcription by Super- Script II (200 U/µl; Invitrogen). Negative controls were prepared without inclusion of the enzyme (non-reverse transcribed controls, RT controls).

Quantitative Real Time PCR (QPCR)

The IL-1RA forward primer was 25 bp, with a G/C content of 48 % and a similar melting temperature (Tm) for forward and reverse primers (59.9°C). The IL-1RA reverse primer was 22 bp, with a G/C content of 59 %. The IL-1RA primer sequence was 5'-CCAGCAAGATGCAAGCCTTCAGAAT-3' for the forward sequence and 5'-CCAGACTTGACACAGGACAGGC-3' for the reverse sequence, and product size was 199 bp. The efficiency of the IL-1RA primer was verified by quantitative real-time PCR (QPCR) (Data not shown). The variation of the quantification cycle number (Cq) was estimated during the exponential phase. A standard curve was generated using serial dilutions of the samples of cDNA (1, 1/5, 1/15, 1/45, 1/135 and 1/405) and plotted using the logarithm of the cDNA dilution versus the average Cq of three replicates.

An efficiency of 80 to 120 % for each set of primers was considered acceptable for further analysis of gene expression.

Quantitative Real-Time PCR (QPCR) and gel analysis

QPCR was carried out with the cDNA prepared from RL95-2 cells as described. For normalization purposes, expression of the reference genes β-actin and B2M were also quantified. The sequence of their primers was as follows: β-actin forward sequence was 5'-CAAGATCATTGCTCCTCCTG-3' and reverse sequence was 5'-ATCCACATCTGCTGGAAGG-3', and product size was 152 bp (Sanchez Lopez et al., 2014b). B2M forward sequence was 5'-TATGCCTGCCGTGTGAACCA-3' and reverse sequence was 5'-GCGGCATCTTCAAACCTCCA-3', and product size was 98 bp (Sanchez Lopez et al., 2014b). SYBR Green Jump Start (Sigma) master mix (containing 10µl SYBR Green, 7µl H2O, 1µl of test or reference gene primers and 1µl cDNA) was added to each well of PCR plate and amplification was performed under the following conditions: 40 cycles of 95◦ for 30 s, 62◦ for 30 s and 72◦ for 30 s. All experiments included RT controls and negative controls (no cDNA). QPCR was performed using Mx3005P QPCR (Stratagene, Waldbronn, Germany) and results were analysed using MxPro QPCR software version 4.01. The amplified QPCR products were sequenced to confirm the identity of the amplified product. The size of the amplicon was confirmed by electrophoresis in a 1% agarose gel

The QPCR data were analysed using the ΔΔCq method. The results were expressed as mean ± SEM. Statistical analysis was performed by using ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered significant.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-1RA was determined in culture supernatants with the commercially available IL-1RA Development ELISA kit (PEPROTECH, London, UK). The ELISA was performed according to the manufacturer's instructions with 100µl of cell-free supernatant.

IL-1RA gene expression Knock down in RL95-2 cells

IL-1RA was knocked down by Accell Human IL1RN siRNA (SMARTpool) (ThermoScientific, Massachusetts, Waltham). In addition, the efficiency of siRNA transfection was assessed using Accell Green Non-targeting siRNA (ThermoScientific, D-001950-01-05, Massachusetts, Waltham) and it was used as negative control (Data not shown). Briefly, IL-1RA siRNA was diluted with Accell Delivery Media (ThermoScientific, Massachusetts, Waltham) to reach a concentration of 1 µM. The growth media were removed from the cells and 100 µl of delivery mix was added to each well of 96-well plate. RL95-2 cells were incubated with IL-1RA siRNA at 37°C with 5 % CO2 for 72 h. The efficiency of IL-1RA siRNA in suppressing IL-1RA's production was measured at both gene and protein levels (Data not shown). The mRNA of knock down cells was assessed by QPCR as described. The sample of knock down cells was assessed by IL-1RA ELISA kit as described.

Transfection of RL95-2 cells with SEAP plasmids containing NF-κB and AP-1 binding regions

The RL95-2 cells were grown in 24-well plates ($2x10^4$ cells) until 70% confluency and transiently transfected with pNifty2-SEAP for NF-κB expression (InvivoGen, Tolouse, France)

or the pNifty3-SEAP for AP-1 expression using X-tremeGENE HP DNA transfection reagent (Roche). Briefly, the media in each well of the 24-well plate were replaced with fresh supplemented media. The mix was prepared using a 1:3 ratio plasmid – transfection reagent in 25 µl of serum-free DMEM/F-12. Supernatant samples were collected and secreted placental alkaline phosphatase (SEAP) was detected using the Phospha-Light™ SEAP Reporter gene assay system (Life Technologies, Applied Biosystems, Paisley, UK) according to the manufacturer's protocol.

Viability assessment of endometrial cells

In order to check the viability of RL95-2 cells treated with either recombinant human IL-1RA or IL-1RA siRNA, RL95-2 cells were grown in 96-well plates until 100 % confluence. The media were replaced with serum free media before they were either treated or not with IL-1RA or IL-1RA siRNA. The cells were then harvested using trypsin-EDTA and collected in 500 µl of media and pelleted by centrifuging at 300 g for 5 min. The cells were then resuspended in 200 µl of PBS and divided in two 5 ml cytometry tubes. One sample was used as an autofluorescence control sample and the other was used for staining with 3 µM propidium iodide (PI; Life technologies, Paisley, UK) and captured immediately. The samples were read in a FACSCalibur cytometer (Rasti et al.) capturing $1x10^4$ events and the percentage of PI positive events (dead cells) was registered. The results were expressed as percentage of live cells and were compared using a one-way ANOVA, with $p < 0.05$ considered significant.

Experimental design

The effect of TLR 3 activation on the production of IL-1RA in RL95-2 cells

To determine whether TLR 3 activation in RL95-2 cells could alter IL-1RA production at the gene and protein level, RL95-2 cells were cultured in 12-well plates and the media replaced with serum-free media before they were either activated or not with Poly I:C at a concentration of 10 μg/ml. IL-1RA gene expression was evaluated by QPCR at 2 and 4 h post TLR3 activation. IL-1RA protein concentration was determined at 1, 2, 4, 6, 8 and 24 h post-TLR3 activation by ELISA as described.

The effect of IL-1RA on binding of trophoblast cells to endometrial cells

In order to determine the influence of the treatment of RL95-2 cells with IL-1RA on the number of trophoblast cells binding to the endometrial cells, RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media before they were either activated or not with IL-1RA at various concentrations (5, 10, 20 and 40 ng/ml) for 4 h. Thirty JAr spheroids were then gently delivered to the endometrial cells in each well and co-incubated for 1 h. Adhesion was assessed as described. In parallel, the viability of RL95-2 cells was assessed by Propidium Iodide staining as described, to exclude detrimental effects of IL-1RA on endometrial cells viability.

To clarify whether the detected response to treatment of RL95-2 cells with Poly I:C was mediated through IL-1RA, IL-1RA expression was knocked down or not in RL95-2 cells using IL-1RA siRNA or IL-1RA negative control siRNA, respectively. RL95-2 cells were treated or not with Poly I:C (10 μg/ml) for 4 h, 30 JAr spheroids were then delivered onto the RL95-2 monolayer and co-cultured for 1 h. Adhesion was assessed as described.

Activation of NF-κB and AP-1 as a result of endometrial TLR activation

The RL95-2 cells were transfected with either the pNifty2 (NF-κB) or pNifty3 (AP-1) plasmids. On the next day, the culture media was replaced with serum-free DMEM/F-12 and the cells were stimulated with 100 ng/ml of flagellin (TLR 5) or 10 μg/ml of Poly I:C (TLR 3) for 4 h. The supernatants were collected and the SEAP was measured with QUANTI-blueTM (InvivoGen). Data were reported as the fold induction of SEAP activity over the non-stimulated control.

Determining the effect of NF-κB and AP-1 on the adhesion of JAr spheroids to the endometrial cells

To assess whether NF-κB or AP-1 pathways could mediate TLR 3-induced impairment of trophoblast adhesion to endometrial cells, the RL95-2 cells were pre-treated or not with the NF-κB and AP-1 inhibitors. The activation of NF-κB and MAPK pathways was inhibited by pre-treating RL95-2 cells with BAY11 (20 μM) and SP600125 (50 μM), respectively, for 1 h. Thereafter, the RL95-2 were either stimulated or not with 10 μg/ml of Poly I:C for 4 h. 30 JAr spheroids were then gently delivered into each well and co-incubated for 1 h at 37°C.

Adhesion was assessed as described. The activity of the inhibitors had been validated before (data not shown) and only their functional dose was applied in this set of experiments.

Statistical Analysis

The results were expressed as mean ± SEM. Statistical analysis was performed using ANOVA (Statistica; Statsoft UK, Letchworth, UK) with Fischer's multiple comparison test. $P < 0.05$ was considered to be significant. All the experiments represented here were performed in three replicates in different days.

Results

JAr spheroids attach and bind to endometrial cells.

After one hour of the Jar spheroids coincubation with the endometrial cells, the JAr spheroids attached and bound firmly to RL95-2 cells (Fig. 1).

Production of IL-1RA was increased in RL95-2 cells in response to Poly I:C.

As shown in Fig. 2A, addition of Poly I:C to RL95-2 cells significantly increased the gene expression of IL-1RA. This effect was observed as soon as 2 h of Poly I:C treatment. A similar effect was observed in the protein level of IL-1RA (Fig. 2B, where there was a clear significant

increase in the IL-1RA levels in response to Poly I:C even after 1 h, despite a slight but significant decline after 4 h.

Addition of IL-1RA to RL95-2 cells significantly decreased percentage of attachment of JAr spheroids to endometrial cells.

The percentage of attachment of JAr spheroids to endometrial cells was significantly suppressed in the IL-1RA treated group compared to control in a dose-dependent manner (Fig. 3A). Reduction of IL-1RA gene expression significantly restored the percentage of attachment of JAr spheroids to endometrial cells in the presence of Poly I:C (Fig. 3B). As shown in Fig. 3C and 3D, recombinant human IL-1RA and IL-1RA siRNA did not have any effect on the viability of RL95-2 cells.

TLR 3 stimulation induced MAPK pathway activity in endometrial cells

The treatment of the endometrial cells with flagellin was able to significantly increase NF-κB activity after 4 h ($P < 0.05$), whereas Poly I:C was unable to increase this activation (Fig. 4A). The treatment of the endometrial cells with Poly I:C was able to significantly induce the activity of AP-1 after 4 h ($P < 0.05$) compared with the non-stimulated control (Fig. 4).

Inhibition of the MAPK JNK pathway affected the binding of the trophoblast spheroids to the endometrial cells

The pre-treatment of the RL95-2 cells with Bay11-7082 was unable to significantly restore the binding of trophoblast cells to endometrial cells in the presence of Poly I:C (Fig. 5). In contrast, addition of SP600125 to endometrial cells significantly recovered the percentage of trophoblast cells attached to RL95-2 cells (Fig. 5).

Discussion

Different factors are required to control the process of implantation, including hormones, cytokines, adhesion molecules and growth factors (Singh et al., 2011). In humans and primates, implantation is known to resemble an inflammatory type response. Different cytokines has been identified in the human endometrium, such as IL-1, whose production was conceived to regulate and control the functions of endometrial cells during the menstrual cycle (Tabibzadeh and Sun, 1992, Tabibzadeh, 1994). IL-1 is a key regulator of the inflammatory response and plays a crucial role in implantation (Bankers-Fulbright et al., 1996). It was shown that IL-1β was expressed in human endometrium (Simon et al., 1993a, Tabibzadeh and Sun, 1992, Kauma et al., 1990) throughout the menstrual cycle and its concentration progressively increased in the secretory phase in comparison to the proliferative phase. This rise was coordinated with the increase in the messenger levels of IL-1 receptor, where IL-1R tI mRNA levels increased significantly in the luteal phase versus the follicular phase (Simon et al., 1993a, Simon et al., 1993b).

411 IL-1RA is also present in the human endometrial epithelial cells (EECs) throughout the entire
412 menstrual cycle (Tabibzadeh and Sun, 1992). IL-1RA concentration increases significantly
413 during the follicular phase versus the early and mid-to-late luteal phase (Simon et al., 1995).
414 Moreover, the IL-1 system was found in the human embryo (De los Santos et al., 1996), and
415 at the maternal-embryonic interface (Simon et al., 1994). Embryonic IL-1 release occurred
416 only when embryos were co-cultured with human EECs (Spandorfer et al.). It is noteworthy
417 that IL-1 produced from human embryos can regulate and increase endometrial receptivity in
418 EEC cells by increasing the expression of α1, α4 and β3 integrins (Simon et al., 1997).
419 Moreover, successful implantation after *in vitro* fertilisation has been correlated to high
420 concentrations of both IL-1α and IL-1β in the culture medium of human embryos (Karagouni
421 et al., 1998). On the other hand, it is well-established that IL-1RA can suppress embryo
422 implantation in mice, by decreasing endometrial receptivity (Simon et al., 1998). Accordingly,
423 the assumption that IL-1RA serves as an anti-implantation factor led us to search for the
424 possibility that TLR 3-induced impairment of trophoblast adhesion to endometrial cells could
425 be mediated through IL-1RA. Hence, we investigated whether TLR 3 activation in RL95-2 cells
426 could alter the gene expression of IL-1RA.
427
428 IL-1RA's gene expression was measured by qPCR and all the steps including RNA extraction,
429 cDNA synthesis and qPCR were performed in accordance to Minimum Information for
430 Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).
431 Our results showed that the gene expression of IL-1RA was increased significantly even after
432 2 h of Poly I:C administration. These data were confirmed by IL-1RA ELISA at the protein level.
433 This is consistent with the findings of Lee and colleagues, who showed that the production of
434 IL-1RA was significantly increased in response to Poly I:C in microglial cells (Lee et al., 2007,

435 Rabehi et al., 2001). In addition, activation of other TLR members such as TLR 4 by LPS has
436 been shown to increase production of IL-1RA in monocytes (Rabehi et al., 2001, Rehani et al.,
437 2009). Although these findings clearly document the stimulatory effect of TLR activation on
438 IL-1RA production, little is known regarding the cellular mechanisms regulating the
439 production of IL-1RA in response to TLR activation. Involvement of MAPK pathway in IL-1RA
440 production has been shown in many studies, where the blocking of p38 and ERK, as subunits
441 of MAPKs, dramatically decreased the production of IL-1RA upon LPS stimulation (Rabehi et
442 al., 2001). This is in agreement with the findings of Rehani et al., in which it was shown that
443 ERK pathway activity is required to augment IL-1RA production upon TLR 4 activation in
444 monocytes (Rehani et al., 2009). Moreover, we were able to show that MAPK pathway activity
445 was significantly increased in endometrial cells in response to Poly I:C. In the same line of
446 evidence it was shown that TLR 3 activation by Poly I:C significantly induced MAPK pathway
447 activity by increasing the production of AP-1 in Fibroblast-like synoviocytes (FLS) (Yoshizawa
448 et al., 2008). Thus, it seems reasonable that induced MAPK pathway activity upon TLR 3
449 stimulation can lead to the observed increased levels of IL-1RA production in response to TLR
450 3 activation. Further investigations are needed to demonstrate this possibility.
451
452 Addition of IL-1RA to RL95-2 cells reduced the adhesion of trophoblast cells to endometrial
453 cells in a dose-dependent manner. Moreover, the inhibitory effect of Poly I:C on spheroid
454 binding was restored consequent to knock down of IL-1RA expression. This data strongly
455 indicates that the observed Poly I:C effect was mediated by IL-1RA. This is consistent with the
456 previous studies, where it was shown that IL-1RA injection in mice prevented embryonic
457 implantation through direct effect on the transformation of plasma membrane of epithelial
458 endometrial cells at the time of implantation (Simon et al., 1998). Moreover, the concept that

IL-1RA could have inhibitory effect on embryo adhesion at the time of implantation is further supported by the findings of Simon and Frances (1995), in which they showed that IL-1RA staining significantly decreased towards the secretory phase, which corresponds to the "window of implantation" (Simon et al., 1995). Together with the experimental data presented here, one can speculate that IL-1RA can lead to inhibition of trophoblast cells binding to endometrial cells *in vivo* and *in vitro*, but the mechanisms through which this alteration happens remain unclear. The current study investigated the effect of IL-1RA on endometrial cells only and further investigation is needed to establish the role of IL-1 system in JAr cells/spheroids and to understand the potential effects of IL-1RA on these cells as well.

After binding to Poly I:C, TLR 3 can activate both the NF-kB and MAPK signaling pathways. In order to clarify the signaling pathways through which TLR 3 activation inhibit trophoblast binding to endometrial cells, both NF-kB and MAPK pathways were blocked using specific inhibitors. Inhibition of NF-κB activity was unable to restore trophoblast cells adhesion to endometrial cells. This is in contrast with the previous finding where it was shown that Bay11-7082 was able to restore the flagellin-induced impairment of the attachment of trophoblasts cells to the endometrial Ishikawa 3H-12 cells (Caballero et al., 2013). It is possible that in our model the TLR 3-mediated reduction of trophoblast spheroid adhesion to the endometrial cells is cell-specific and could signal through a different route such as MAPK pathway. Treatment of RL95-2 cells with the MAPK JNK inhibitor SP600125 recovered the spheroid adhesion to endometrial cells in the presence of Poly I:C. This data confirmed the inhibitory role of MAPK JNK pathway on trophoblast spheroid adhesion to endometrial cells in RL95-2 cells. Hence, it might be logical to believe that MAPK pathway could mediate the TLR 3-induced production of IL-1RA, which in turn leads to the impairment of trophoblast cells'

attachment to endometria cells (Fig 6). Further investigation is required to establish the role of MAPK pathway in this system. In this regard, blocking MAPK pathway and investigating its effect on the Poly I:C-induced IL-1RA production would be worth trying. The involvement of MAPK pathway in the TLR 3-mediated inhibition of trophoblast cells' adhesion to endometrial cells has been shown before in our previous study (Montazeri et al., 2015), where it was found that MAPK mediated TLR 3-induced impairment of actin polymerization, cluster of differentiation (CD98) and β3 integrin expression, which may result in impairment of trophoblast adhesion.

Since IL-1 is such a potent inflammatory cytokine, it is critical that its biological effects be precisely controlled. In this regard, IL-1RA acts as a regulator of IL-1 biological effects and when it binds to IL-1R tI on the cell surface, it blocks the IL-1 signal transduction (Bankers-Fulbright et al., 1996). The balance of the IL-1/IL-1RA levels is crucial. Indeed, it is well documented that the relative levels of IL-1 and the endogenous IL-1RA correlate with the pathogenesis of many diseases and an excess amount of IL-1, for instance, can develop inflammatory and autoimmune diseases such as diabetes and rheumatoid arthritis (Bankers-Fulbright et al., 1996, Arend, 2002). In addition, increased levels of IL-1RA have been found in the circulation of patients with a variety of inflammatory, infectious, and post-surgical conditions (Arend et al., 1998). This indicates the importance of hepatic production of IL-1RA as an acute phase protein, which diffuses into the tissues and influences the local ratio of IL-1RA to IL-1 (Gabay et al., 1997). Accordingly, treatment of these diseases has been carried out by injection of recombinant IL-1RA protein or using gene therapy approaches (Arend, 2002). An appropriate ratio of IL-1 to IL-1RA is also pivotal to initiate and maintain successful implantation at the local fetal-maternal interface (Huang et al., 2001), and as shown in mice

and the current study, increased levels of IL-1RA can interfere with the process of trophoblast cells binding to endometrial cells and may result in implantation failure. This can be due to the fact that in the presence of excess amount of IL-1RA, the stimulatory effect of IL-1β on endometrial receptivity (Simon et al., 1997) is blocked and as a result of that, the adhesion of embryo to endometrial cells is impaired. In the same line of evidence, the fact that IL-1RA expression significantly decreased in the secretory phase (Simon et al., 1995), when embryo implantation is taking place suggests the existence of specific inhibition of IL-1RA production at the time of implantation, which facilitates IL-1 pre-implantation actions on endometrial receptivity. The window of implantation is the time frame when the endometrium changes for the arrival of the embryo. For this reason, a safe environment should be guaranteed. If the innate immune system is activated at this time, the uterine tissue is able to respond actively. On one hand, the response will defend the maternal tract from a potential infection, but on the other hand this defense strategy might result in adversely affecting implantation of the embryo.

Approximately 35% of infertile women are afflicted with post-inflammatory changes of the reproductive organs, most of which result from infection in the FRT (Novy et al., 2008). Although, no data is available to support what proportion of these infertility cases are virus-induced. As mentioned before, a strong association between HSV and CMV infection and female infertility, spontaneous abortion and congenital defects has been found (el Borai et al., 1997, Medvedev et al., 2009, Yang et al., 1995, Kalu et al., 2015, Rasti et al., 2015, Vasileiadis et al., 2003, Weichert et al., 2010). Both HSV and CMV infection recognition with TLR 3 has been shown before (Matsumoto et al., 2011). This evidence in conjunction with the data obtained from our work that maternal TLR3 activation impairs trophoblast cells'

adhesion to endometrial cells, show the possibility of the involvement of maternal HSV and CMV infection in the failure of embryo implantation. Although, no concrete evidence may exist to pinpoint the association between HSV and CMV infection and embryo implantation failure in human, the effect of CMV infection on embryo implantation has been determined in mice, where the inoculation of maternal CMV significantly reduced embryo implantation rates (Neighbour, 1976). Our findings raise the possibility of clinical significance of screening and treatment for viral infections among pre-pregnant women to identify and treat the high-risk population for virus-induced implantation failure. This will improve not only the embryo implantation rate in ART techniques but also will deepen our understanding of different factors involved in endometrial receptivity.

Many factors produced by the endometrium during the window of implantation have been proposed as molecular markers of endometrial receptivity, such as LIF and mucin 1 (Sharkey and Smith, 2003), but little attention has been paid to their application in the treatment of infertility. In the current study the discovery that increased levels of IL-1RA upon TLR 3 activation directly impair trophoblast adhesion to endometrial cells could be used as a significant diagnostic and therapeutic tool for the treatment of viral-induced infertility cases.

To conclude here, we report our findings that IL-1RA mediated TLR 3-induced impairment of trophoblast cells adhesion to endometrial cells *in vitro*. This finding demonstrates the potential clinical application of IL-1RA as an infertility diagnostic marker. Moreover, based on the novel data obtained in the current study, blocking and regulating the MAPK pathway by its inhibitors can be used as a new strategy to prevent and treat virus-induced infertility cases in ART techniques.

Author's roles

Each author made substantial contributions to the design of the study, the interpretation of the data and the drafting and revising of the submitted manuscript. A.F. and M.M. designed the original study and M.M. designed and operated the experimental work. J.A.S.L and N.M. also participated on the design of the study. M.M., I.C. J.A.S.L., N.M., S.E. and A.F. all contributed to the follow-up study design, data collection and manuscript preparation. All authors have seen and approved the final submitted manuscript.

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Conflict of interest

None declared.

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Figure legends

Figure 1. JAr spheroids attach and adhere to endometrial cells.

Once the JAr spheroids were formed on the shaker, they were gently transferred onto each well of confluent RL95-2s in 12-well plates, and the co-culture was maintained in DMEM-F12 HAM and incubated for 1 h at 37°C. The images of JAr spheroids and RL95-2s co-culture were then captured by an inverted fluorescent microscope.

Figure 2. The effect of TLR 3 stimulation on IL-1RA production in RL95-2 cells.

RL95-2 cells were treated with 10 μg/ml of Poly I:C for various time points. Firstly, the gene expression of IL-1RA was assessed by QPCR (A). Secondly, the IL-1RA production at the protein level was investigated by IL-1RA ELISA (B). The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare

the gene expression and production of IL-1RA at each time interval. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

Figure 3. The effect of IL-1RA on trophoblast cells binding to endometrial cells.

RL95-2 cells were treated with IL-1RA at various concentrations (0, 5, 10, 20 and 40 ng/ml) for 4 h. The effect of IL-1RA was then investigated on the trophoblast adhesion to endometrial cells (A). RL95-2 cells were then transfected by IL-1RA siRNA at a concentration of 1 μM for 72 h. They were then treated by Poly I:C (10 μg/ml) for 4 h and the influence of IL-1RA knock down on trophoblast binding to RL95-2 cells was assessed (B). The viability of RL95-2 cells was determined after their treatment with different concentrations of IL-1RA (0, 5, 10 and 20 and 40 ng/ml) (C). In addition, the effect of addition of IL-1RA siRNA to RL95-2 cells on the viability of endometrial cells was assessed (D). The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of attached JAr spheroids to endometrial cells in each group. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

Figure 4. Activation of NF-κB and AP-1 in RL95-2 cells after TLR stimulation.

RL95-2 cells were transfected with the pNifty2 (NF-κB) and pNifty3 (AP-1) plasmids containing a secreted alkaline phosphatase (SEAP) reporter. The cells were stimulated with the ligands for TLR 5 (100 ng/ml Flagellin) and TLR 3 (10 μg/ml Poly I:C) for 4 h. SEAP production was measured and results represented as fold of NF-κB and AP-1 activation with untreated control. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the fold of NF-κB and AP-1 production in each group. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

Figure 5. Pre-treatment of the RL95-2 cells with AP-1 inhibitor restored the TLR 3-induced reduction of JAr spheroid adhesion.

RL95-2 cells were pre-treated with Bay11-7082 (20 μM) and SP600125 (50 μM) for 1 h. The cells were stimulated with TLR 3 ligand (10 μg/ml Poly I:C) for 4 h. 30 JAr spheroids were then delivered and co-cultured with the endometrial cells for 1 h. The plate was rinsed and the results are expressed as the percentage of attached spheroids. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids in each group. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

Figure 6. A mechanistic pathway of TLR 3-induced impairment of trophoblast adhesion to endometrial cells.

A schematic demonstration of TLR 3 signalling pathway, showing the potential involvement of mitogen-activated protein kinases (MAPK) pathway in TLR 3-induced IL-1RA production,

798 alteration in cell cytoskeleton's arrangement and expression of adhesion molecules (cluster
799 of differentiation (CD98) and beta3 integrin), which in turn leads to inhibition of trophoblast
800 cells' adhesion to endometrial cell (Montazeri et al., 2015).
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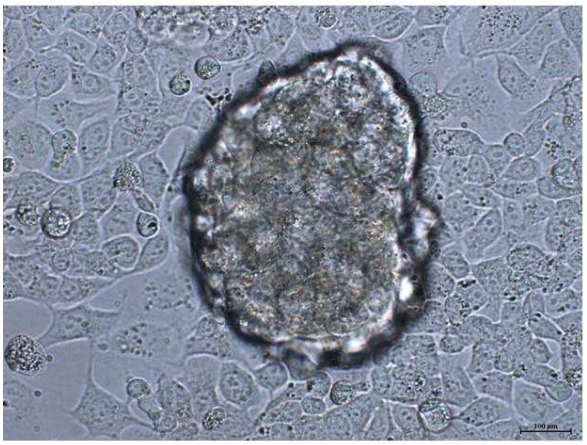

Figure 1

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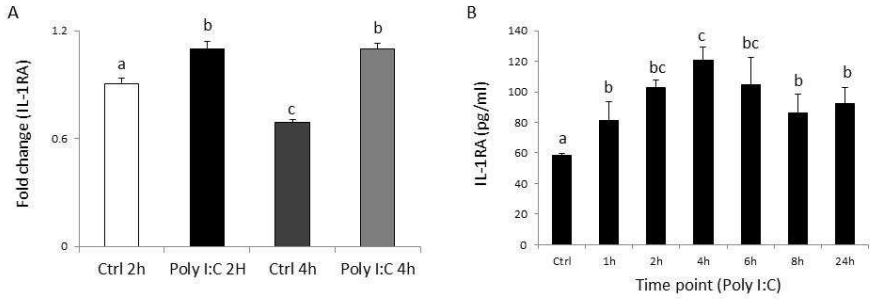

Figure 2

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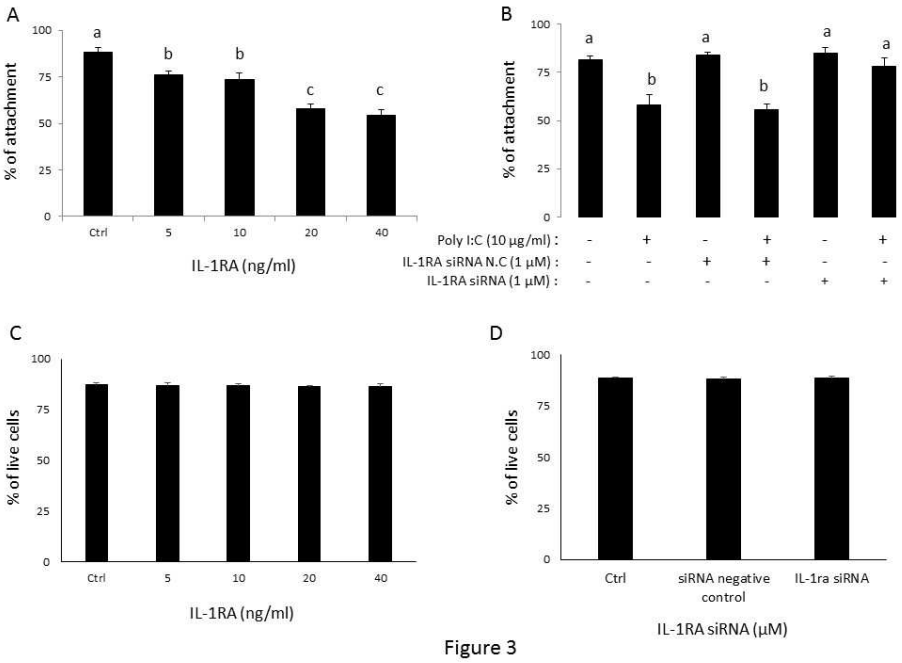

Figure 3

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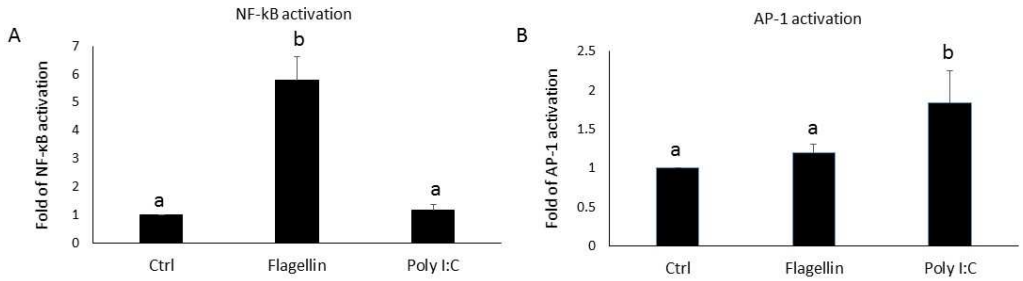

Figure 4

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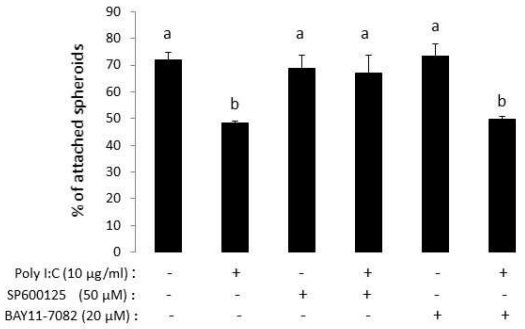

Figure 5

811
812

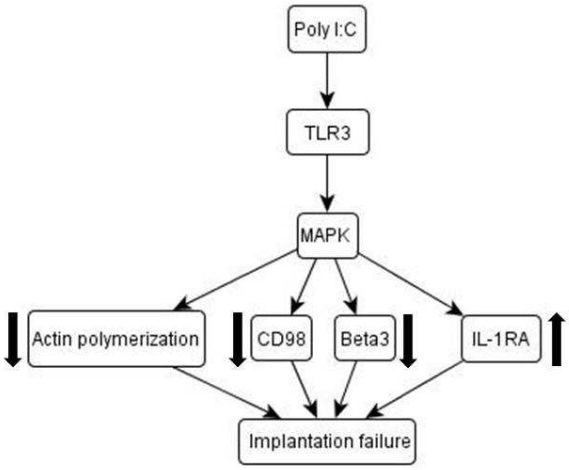
Poly I:C
TLR3
MAPK
Actin polymerization
CD98
Beta3
IL-1RA
Implantation failure

Figure 6

813